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Original Paper

Overexpression of Ribosomal Proteins L4 and L5 and the Putative Alternative Elongation Factor PTI-1 in the Doxorubicin Resistant Human Colon Cancer Cell Line LoVoDx^R

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A better understanding of the regulatory network underlying cellular drug resistance and stress response may be helpful to overcome the phenomenon of therapy-induced cross-resistances against a variety of antineoplastic agents. Two new powerful molecular techniques, mRNA differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) and subtractive suppressive hybridisation were applied for the comparative analysis of the gene expression profile of a doxorubicin resistant and its corresponding sensitive parental colon carcinoma cell line (LoVo H67P). DDRT-PCR generated partial cDNAs from the doxorubicin resistant, sensitive and stress (dexamethasone, doxorubicin, cadmium chloride or heat) exposed sensitive cells, were size-separated on polyacrylamide gels. The expression patterns of more than 9000 bands of the resistant, sensitive and stressed sensitive cell populations were identical by more than 95%. Of the differentially expressed mRNAs, 20 cDNA fragments were reamplified after isolation from the gel, used as probes for Northern blot analysis to verify their differential expression and sequenced after cloning. Among the differentially expressed cDNAs, homologies of 96% and 87%, respectively, were found to the human proto-oncogene *PTI-1* and the human ribosomal protein L4. Subtractive suppressive hybridisation revealed overexpression of the ribosomal protein L5 in the doxorubicin resistant line. These data point to the control of gene expression at the translational level as an important mechanism involved in cellular stress response.

Key words: differential display, subtractive suppressive hybridisation, cellular stress response, proto-oncogene *PTI-1*, ribosomal proteins, ribosomal stress sensoring
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INTRODUCTION

In vitro, predominant mechanisms of cellular drug resistance, such as active drug export mechanisms, have been identified. However, there is only a poor correlation of these *in vitro* data (e.g. overexpression of *P*-glycoprotein, Pgp) with clinical observations and to the therapeutic response. The exposure of cells to a variety of antineoplastic agents can be regarded as a general 'stress' stimulus for these cells. Therefore, it is likely that, apart from the development of a more or less drug specific resistance, other more general mechanisms of resistance may occur which may involve a broader spectrum of anticancer drugs.

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This assumption prompted us to look for a possible superimposed mechanism of drug resistance interfering with known components of the multiple drug resistance (MDR) phenotype, e.g. Pgp. In Northern blot experiments comparing a doxorubicin resistant and the respective sensitive cell line (LoVo H67P), we found that only the resistant cell line expressed hsp90β. In further experiments, using immunoprecipitation and antisense technology (antisense oligonucleotides directed against Pgp and hsp90β used either alone or in combination), a co-operation of hsp90β and Pgp could be related to the degree of doxorubicin resistance [1]. Based on these findings, we became interested in the more general mechanisms underlying cellular drug resistance and cellular heat shock response and used the aforementioned doxorubicin sensitive and resistant colon cancer cell lines LoVo H67P and LoVo Dx^R as a model.

The recently developed reverse transcriptase differential display polymerase chain reaction (DDRT-PCR) allows the rapid and sensitive comparison of mRNA expression of different cell populations. The basic strategy for differential display is a reverse transcription of the cellular mRNA pool followed by an amplification using a set of anchored oligo (dT) primers and of arbitrary decamers [2] or a non-anchored PCR using two arbitrary decamers [3]. In the present work, we describe the application of this powerful technique in combination with an improved method of subtractive hybridisation, subtractive suppressive hybridisation. Subtractive suppressive hybridisation allows the preferential amplification of differentially expressed cDNAs in two cell populations by suppression of sequences common in both populations which can be separated after hybridisation. Both methods were used to analyse genes which are differentially expressed in the doxorubicin resistant colon carcinoma cell line LoVo Dx^R and the respective sensitive parental line. For a further restriction of differentially expressed RNAs to those which may be involved in drug resistance, doxorubicin sensitive cells after exposure to stress factors, such as dexamethasone, doxorubicin, cadmium chloride or elevated temperature, were included in this DDRT-PCR comparison. As previously shown [1], these stress stimuli can induce the expression of proteins which are thought to be involved in Pgp-mediated doxorubicin resistance.

MATERIALS AND METHODS

Cell culture

The cell lines shown in Table 1 were obtained either from the ATTC (Rockville, Maryland, U.S.A.) KB 3-1, SW 480, SW 48, WiDr, HT 29; or the DSM (Braunschweig, Germany) KB V1, CX-1; or in the case of LoVo H67P and LoVo Dx^R from Dr Rivoltini (Milan, Italy) and ARH 77 and ARH D60 from Dr Bellamy (Tucson, Arizona, U.S.A.). Cells were cultured at 37°C in CLICKS/RPMI medium containing 10% heat-inactivated fetal calf serum (FCS). For differential display analysis or subtractive suppressive hybridisation, 5×10^6 cells were seeded in 80 cm^2 cell culture flasks and grown for 18 h. For stress treatment, doxorubicin sensitive LoVo H67P cells were exposed to either 43°C for 1 h, 0.5 μg/ml doxorubicin for 1 h, 200 μM cadmium chloride for 1 h or 1 μM dexamethasone for 3 h [5–9].

RNA isolation, differential mRNA display and reamplification of differentially expressed RT-PCR products

The untreated and stress exposed cell populations were washed twice directly in the culture flasks with phosphate buffered saline and then treated with guanidiniumthiocyanate (GTC). Total RNA was isolated according to the method of Chirgwin and associates [10]. For cDNA synthesis, 0.2 μg of RNA was subjected to a reverse transcription reaction using 2.5 μM of one of the 12 T_{11} MN (M: A, C or G; N: A, C, G or T) anchored primers, 20 μM dNTPs and 200 U M-MLV (Gibco/BRL, Germany) at 37°C for 60 min. This resulted in 12 cDNA pools for each cell population. The subsequent PCR reaction was performed using 2.5 μM of a combination of two arbitrary primers published by Bauer and colleagues [11] in a buffer containing 70 μM dNTPs, 1.5 mM $MgCl_2$ and 1 U Tfl DNA polymerase (Biozym, Germany) for 40 cycles (94°C, 30 sec; 40°C, 30 sec; 72°C, 30 sec) in a 30 μl reaction volume. This resulted in a set of approximately 9000 cDNA fragments for each cell population.

After separation on a 6% polyacrylamide gel, the PCR product bands were visualised using a previously published silver staining protocol [12]. Differentially expressed cDNA fragments were directly excised from the silver stained gels, vacuum dried, resuspended in 50 μl PCR buffer (50 mM TRIS, pH 9.0, 20 mM $(NH_4)_2SO_4$ 1.5 mM $MgCl_2$) and heated for 30 min at 94°C. The following reamplification of the isolated cDNA fragments was performed according to the first PCR protocol using the respective primer combination, but at 150 μM and an annealing temperature of 42°C instead of 40°C as in the preceding PCR. Reamplification products were analysed either on 2% agarose gels stained with ethidium bromide or on 6% polyacrylamide gels followed by silver staining. Subtractive suppressive hybridisation was performed using the PCR-Select™ cDNA Subtraction Kit

Table 1. *Expression of PTI-*1 *and the human elongation factor EF* 1α *in different normal human tissues and in various human cell lines from haematological and solid tumours*

	PTI-1	EF-1α
Heart	–	++
Brain	–	++
Placenta	–	++
Lung	–	++
Liver	–	++
Muscle	–	++
Kidney	–	++
Pancreas	–	++
Colon	–	++
ARH-77	+	++
ARH-D60	+	++
Raji	–	nd
HL-60	–	nd
K-562	–	nd
Molt-4	++	nd
G361	+	nd
A549	–	nd
KB 3-1	+++	+
KB V1	+	+
SW-480	+	+
SW-48	+++	+
WiDr	++	++
HT-29	++	++
CX-1	+	++
LoVo	++	+
LoVo Dx^R	+++	+
LoVo dAc	–	++
LoVo Vin	–	++
LoVo cPt	–	++

nd, not determined; –, no expression; +, weak expression; ++, expression; +++, high expression. LoVo Dx^R, doxorubicin resistant; LoVo dAc, actinomycin-D resistant; LoVo Vin, vincristine resistant; LoVo cPt, cisplatin resistant; ARH, multiple myeloma; ARH D60, doxorubicin resistant variant; Raji, Burkitt's lymphoma; HL-60, promyelocytic leukaemia; K-562, chronic myelogenous leukaemia; Molt-4, lymphblastic leukaemia; G361, melanoma; A549, lung carcinoma; KB, epidermoid carcinoma; KB V1, doxorubicin resistant variant; SW 480, SW-48, WiDr, HT-29, CX-1, LoVo, colorectal adenocarcinoma. PTI-1 and EF-1α are identical in more than 90% of their 3′ part, specific detection of PTI-1 expression is possible using a probe against the extended PTI-1 specific 5′ part of the sequence [4]. For cell lines Raji, HL-60, K-562, Molt-4, G361 and A549, as well as for the tissues indicated, the respective Multiple tissue Northern Blots MTN I and the Human Cancer Cell Line Blot (Clontech, Heidelberg, Germany) were used.

supplied by Clontech (Heidelberg, Germany), according to the manufacturer's instructions.

Slot blot and Northern blot analysis

For a rapid screening of cloned cDNA fragments from the DDRT-PCR reactions, in respect of a differential expression so uncovering PCR related artefacts, a slot blot hybridisation was performed. For this purpose, the sub-cloned PCR fragments within the cloning vector were slot blotted. Subsequently they were probed with a labelled cDNA generated by cDNA synthesis from total RNA preparations from the respective cell lines (doxorubicin resistant, sensitive and the respective stressed sensitive cell populations) using 5 μg total RNA and an oligo dT primer [13]. cDNA probes were labelled with DIG-dUTP according to the manufacturer's instructions (Boehringer Mannheim, Germany). After the slot blot procedure, membranes (HybondN$^+$, Amersham) were hybridised in 5 × SSC (standard saline cititrate) [14], 5 × Denhardts, 1% sodium dodecyl sulphate (SDS) at 65°C for 12 h with one of the labelled cDNAs. Prehybridisation was performed in the same solution without the probe. After hybridisation, membranes were washed in 2 × SSC, 0.1% SDS and subsequently in 0.1 × SSC, 0.1% SDS at 65°C. Hybridised probes were visualised by chemiluminescence using CDP-star as a substrate following the manufacturer's protocol (Boehringer, Germany). The membranes were then exposed to Kodak XAR films for between 2 and 8 h.

For Northern blot analysis, total RNA from the cell lines was isolated by caesium chloride gradient centrifugation. Total RNA (25 μg) was separated on denaturating agarose gels [14], transferred to nylon membranes and used for Northern blot analysis with the differentially expressed cDNA RT-PCR fragments previously verified by slot blot analysis.

Northern blot analysis to analyse the expression of PTI-1 or the closely related EF-1α was performed using total RNA from the indicated cell lines or the Clontech Multiple Tissue Northern Blot I. PTI-1 specific probes were generated with the primer pair indicated by Shen and associates [4], the probe for EF-1α, which also identifies PTI-1, was generated in a PCR reaction using the following primer pair:

5′-primer: ATC AAT CGC ACA ATG TGG GC
3′-primer: GCG CTT ATT TGG CTT GGA TG

Cloning and sequencing of cDNAs

After reamplification of excised differential RT-PCR products, cDNA fragments were ligated into the vector PCR™II using the TA cloning kit according to the instructions of the manufacturer (Invitrogen, Heidelberg, Germany). For the ligation into the aforementioned vector, adenosine residues were added at the 3′-ends of the PCR products by incubation in PCR buffer in the presence of Taq DNA polymerase at 70°C for 10 min. Ten randomly chosen bacterial colonies from each transformation assay were cultured. Plasmid preparations were performed using the Magic Wizzard plasmid preparation kit (Promega, Heidelberg, Germany). After insert control, plasmids were sequenced using the cycle sequencing method on an Applied Biosystems 373A automated sequencer. Cycle sequencing was performed according to manufacturer's instructions. The obtained sequences were subjected to sequence alignment with the NCBI GenBank using the BCM Search Launcher and Basic Blast Search (Blastn).

RESULTS

The stringency of the PCR reactions was empirically adjusted to obtain an average of 50 amplification products per primer combination separated in one lane by comparing different PCR parameters (e.g. nucleotide concentration as well as temperature and time profile). The PCR products separated on polyacrylamide gels varied in size from 50 to 1500 bp (Figure 1). Banding patterns of the doxorubicin resistant, sensitive and stressed sensitive cells were more than 95% identical, as shown by mobility of the PCR fragments after gel electrophoresis (Figure 1). For the identification of differentially expressed genes in doxorubicin resistant, sensitive and stressed sensitive cells, cDNA fragments with an apparent differential expression generated during the first PCR reaction were isolated from the polyacrylamide gel, reamplified by the original primer combination and analysed either on agarose or polyacrylamide gels. A total of 20 differentially expressed cDNA fragments were cloned and sequenced. Databank comparison revealed that 10% of the identified sequences were identical to known complete mRNA sequences. Homologies to various expressed sequence tags (ESTs) were not further considered in this study; mostly, homologies to ESTs were only over shorter stretches (40–50 nucleotides). Among the differentially expressed PCR fragments found by DDRT-PCR, a product with 94% homology to the recently discovered proto-oncogene *PTI*-1 [4] and a product with 87% homology to the human ribosomal protein L4 [15] were identified (Figure 2).

Northern blot analysis (Figure 3) confirmed differential expression of 5/20 of the identified mRNAs, whilst 15/20 were false positive. PTI-1 and human ribosomal protein L4 (Figure 3, lanes A and B) had higher expression in the resistant cell line, whereas two other identified mRNAs were only expressed in the sensitive cell line (Figure 3, lanes D and E). This finding demonstrates that resistance does not always result in a gain of expression/function, but can also be due to a loss of expression/function, thereby preventing the activation or the transport of a drug. The PCR fragments shown in Figure 3, lanes D and E were not further characterised in this study.

Subtractive suppressive hybridisation [16] which, in our hands, leads to a significantly higher number of informative sequences (40% false positive versus 75% false positive using DDRT-PCR) resulted in the identification of a second ribosomal protein L5 in 1 of 3 differentially expressed PCR fragments; the two others were MAGE 3 and S100P. L5 was expressed in the doxorubicin resistant cell line LoVo D_X^R, but not in the sensitive one (LoVo H67P) as confirmed by RT-PCR.

The expression pattern of PTI-1 was analysed in different normal human tissues (Clontech Multiple Tissue Northern Blot I) and in a variety of human cell lines derived from haematological or solid tumours by Northern blot analysis (Table 1). Expression of PTI-1 could not be detected in the normal tissues investigated, but the homologue EF 1α, comprising approximately 90% of the 3′ sequence of PTI-1, was present in all these tissues. PTI-1 expression, using a probe generated with PTI-1 specific primers [4], was seen in most of the investigated cell lines, predominantly in cell lines from

solid tumours. The highest expression was found mainly in colon tumour cell lines (Figure 4).

DISCUSSION

The aim of our investigation was the identification of genes which may be involved in the network underlying cellular drug resistance and stress response. The understanding of this regulatory network may be helpful in elucidating common steps or pathways resulting in the evolution of drug resistant tumour clones in cancer chemotherapy. Some defined resistance mechanisms—at least in cell culture—are well characterised and studied in detail. A well characterised example is the Pgp-related MDR positive phenotype in which drug resistance is thought to be related to the transmembrane extrusion of the drug out of the cell. However, little is known about the complexity of the development of cross-resistances during chemotherapy and the relationship of drug resistance and cellular stress response.

mRNA differential display is a sensitive technique for rapidly analysing differentially expressed genes in closely related cell populations. There are two slightly different approaches for the application of this strategy. The protocol of Liang and Pardee [2] uses anchored oligo dT primers comprised of 11 or 12 dT residues and two additional bases at the 3′ termini of the primers to provide specificity for cDNA synthesis. An anchored downstream primer is also selected for the subsequent PCR reaction combined with arbitrary 10mer primers. However, this approach often

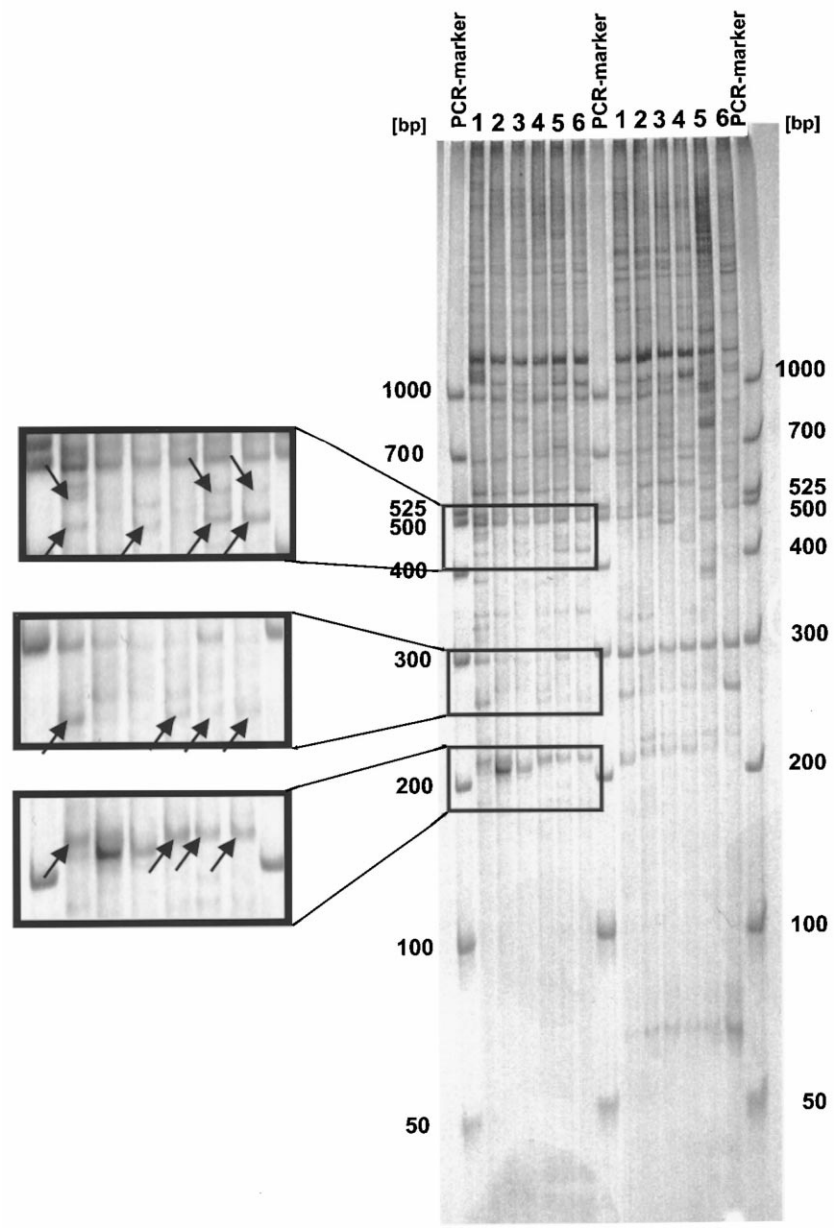

Figure 1. Comparative differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) analysis of the gene expression pattern of doxorubicin resistant and sensitive, as well as stressed sensitive colon carcinoma cell line LoVo. Differentially expressed cDNA fragments are marked in the inserts. The apparent identical banding pattern (approximately 95%) shows that most of the products are expressed in all populations (sensitive, doxorubicin resistant and stress treated sensitive cell populations). Lane 1, LoVoDx^{R}; lane 2, LoVo; lane 3, LoVo heat stressed; lane 4, LoVo + dexamethasone; lane 5, LoVo + doxorubicin; lane 6, LoVo + cadmium chloride.

human elongation factor EF-1α mRNA, (identity = 146/154 (94%))

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49
GCACAATGTGGGCTTCAATGTCAAGAATGTGTCTGTCAAGGATGTTCGCCGTGGCAACGTTGCTGGTGACAGCAAAAATGACCCACCAATGGAAGCAGCTG
| |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||||    ||||||||||||||||||
GGACAATGTGGGCTTCAATGTCAAGAATGTGTCTGTCAAGGATGTTCGTCGTGGCAACGTTGCTGGTGACAGCAAAAATGATTTACCAATGGAAGCAGCTG
216


GCTTCACTGCTCAGGTGATTATCCTGA 175
|||||||||||||||||||||||||||
GCTTCACTGCTCAGGTGATTATCCTGA 342
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human ribosomal protein L4 (RPL4) mRNA, (identity = 63/72 (87%))

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115 GATCAATCGCAGAGTCCTAAAGAAGAACCCACTGAAAAACTTGAGAATCATGTTGAGCTTTTGCCCTTCTGC 44
    ||||  |||||||||||||||||||||||||||||||||||||||||||||||||||||||    |   ||| | |||
37  GATCCATCGCAGAGTCCTAAAGAAGAACCCACTGAAAAACTTGAGAATCATGTTGAAGCTAAACCCATATGC 108
```

Figure 2. Sequence alignment of differentially expressed cDNA fragments to known mRNA sequences. Emphasis was put on homologies to known full-length mRNAs and not to expressed sequence tags (ESTs). Query sequence is in *italic*.

results in the identification of 3′ untranslated regions and not of coding sequences, which hampers the search for structural motifs of known protein families in unknown sequences. The approach of Haag and Raman [3] also uses an anchored cDNA synthesis, but two arbitrary primers are utilised for the PCR amplification, thus resulting in cDNA fragments probably located more in the 5′ region of a given but unknown RNA sequence.

DDRT-PCR has rapidly become a popular procedure for the detection and identification of differentially expressed genes [2, 4, 17] and has been successfully applied for the identification of several genes which are important for cellular regulation. Routinely, mRNA display is visualised by autoradiography after incorporation of [^{33}P]dATP or [^{35}S]dATP during the PCR reaction. However, there are non-radioactive alternatives to this approach allowing for a direct visualisation of the PCR products in the polyacrylamide gel which enables precise excision of the differentially expressed PCR products for reamplification and other manipulations.

In some recent reports, it has been shown that heat shock protein expression is not non-specifically elevated after chemotherapy, but that several heat shock proteins are connected in a specific manner to well-defined mechanisms of drug resistance [1]. However, there are only preliminary data concerning the mechanistic basis of this relationship. In transfection experiments using an hsp27 expression vector introduced in Chinese hamster lung fibroblasts, Huott and colleagues [18] were able to demonstrate a cross-resistance against the MDR-related drugs vincristine, doxorubicin, daunorubicin, colchicine and actinomycin D.

Our approach of not only comparing doxorubicin resistant and sensitive cells but also stressed sensitive cells was successful by restricting differences in the RNA pools of the doxorubicin resistant and the sensitive colon carcinoma cell

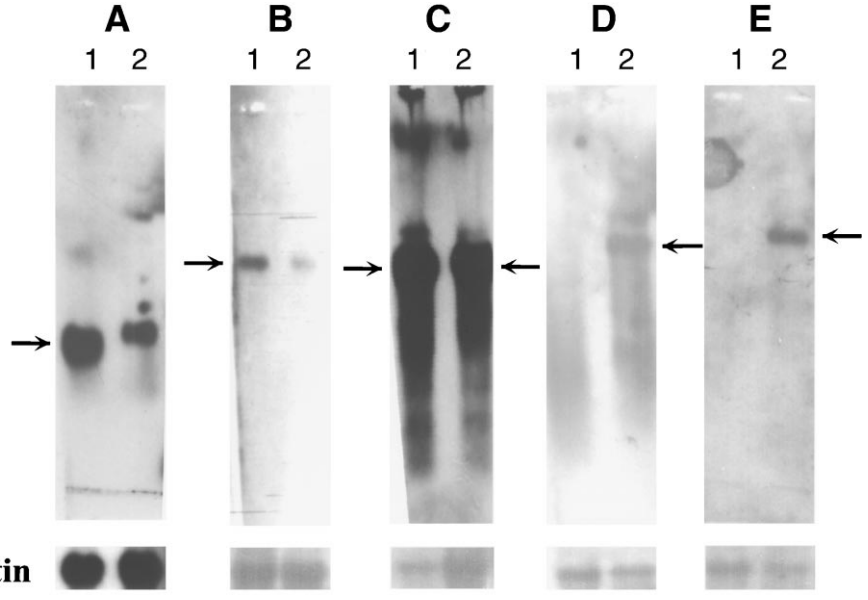

Figure 3. Northern blot analysis of differentially expressed mRNAs. Lanes A and B, arrows indicate higher expression in the resistant cell line; lane C, arrows indicate equal expression in both cell lines; lanes D and E, arrows indicate expression only in the sensitive cell line. 1, doxorubicin resistant line LoVo Dx^{R}; 2, sensitive parental line LoVo. β-actin was used to control RNA load. Sequence alignment as shown in Figure 2 shows homologies of the product in lane A to PTI-1 and in lane B to the ribosomal protein L4. The product in lane C is an example of a false positive polymerase chain reaction fragment. Products in lanes D and E which are expressed in the sensitive and not in the resistant LoVo cell line (loss of expression) were not further analysed in this study.

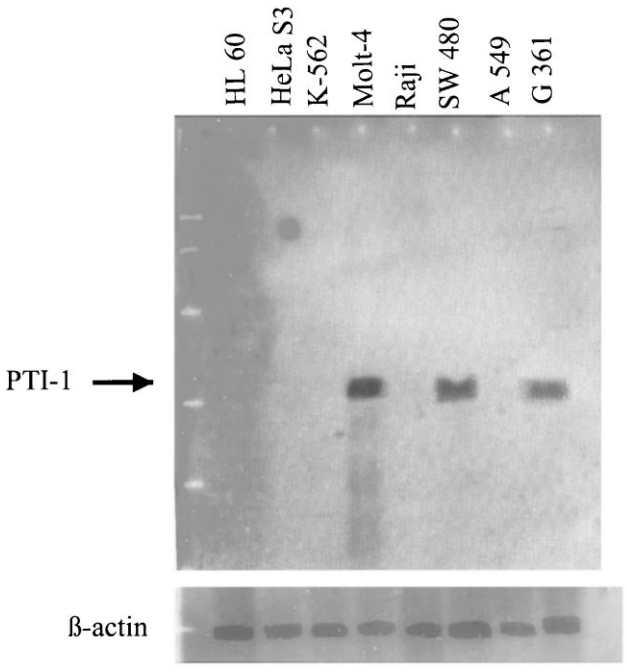

Figure 4. Northern blot analysis of the expression of PTI-1 in different cancer cell lines (Multiple Cancer Cell Line Blot, Clontech, Heidelberg, Germany). A DIG-dUTP labelled probe was generated as described in Materials and Methods using primers published by Shen and colleagues [4].

line to drug and stress related alterations. This approach was based on the finding that stress treatment of the doxorubicin sensitive colon carcinoma cell line LoVoH67P resulted in the induced expression of hsp90β, a protein found in the doxorubicin resistant variant LoVoDx^R but not in the doxorubicin sensitive parental line [1]. Based on these findings, the doxorubicin sensitive/resistant couple of the colon cancer cell line LoVoH67P was used as a model to search for putative superimposed mechanisms leading to a drug resistant and possibly therapy refractory phenotype.

Using highly sensitive comparative methods, a product with 96% homology to the recently discovered oncogene *PTI*-1, a EF 1α homologue [4] and a product with 87% homology to the human ribosomal protein L4 [15] were identified. A second ribosomal protein, L5, was found to be expressed in the doxorubicin resistant colon cancer line but not in the sensitive parental one, using subtractive suppressive hybridisation.

The finding that two ribosomal proteins (L4 and L5) and the putative alternative elongation factor PTI-1 (sharing 96% homology with the elongation factor EF 1α) are only expressed in the doxorubicin resistant cell line may indicate an involvement not only of the transcription but also the translation machinery in cellular stress sensoring and response. In this context, it is interesting that a comparison of 5′ untranslated leader sequences of stress induced eucaryotic mRNAs [19] revealed a high sequence homology for a 150 nucleotides long stretch of the respective RNAs. This sequence might represent an internal ribosome entry site (IRES) as discussed by the authors. Taken together with stress induced changes in the stoichiometry of ribosomal proteins (e.g. L4 and L5) and alternative elongation factors (e.g. PTI-1), these IRES sequences may allow the translation of a specific array of proteins after stress exposure to trigger an adaptive cellular stress response. This stress response can be involved in the initial development of drug resistance, followed by a more specific selection of drug resistance phenotypes depending on the ongoing selection in relation to a specific drug regimen.

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